Calcium-Induced Associations of the Caseins: A Thermodynamic Linkage Approach to Precipitation and Resolubilization

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Calcium-induced changes in protein solubility play a role in a variety of important biological processes including the deposition of bone and dentin and the secretion of milk. The phenomena of salt-induced (calcium) precipitation of proteins (salting-out), and the resolubilization of these proteins at higher salt concentrations (salting-in) have been studied and quantitated using an approach based on the concepts of Wyman's thermodynamic linkage. Salting-out has been described by a salt-binding constant, k_1, the number of moles of salt bound per mole of protein, n, and S_1, the fraction soluble at saturation of n; salting-in has been described by corresponding constants k_2, m, and S_2. Analysis of salt-induced solubility profiles was performed using nonlinear regression analysis. Results of calcium-induced solubility profiles of two genetic variants of α_{s1}-casein (α_{s1}-A), (α_{s1}-B), and β-casein C (β-C) at 37°C, where hydrophobic interactions are maximized, showed no salting-in behavior and for salting-out, yielded k_1 values of 157, 186, and 156 liters · mol^{-1} and n values of 8, 8, and 4, respectively. The values of k_1 can be correlated with the apparent association constant for calcium binding to casein, while the values of n can be correlated with the number of calcium binding sites of the respective caseins. At 1°C, where hydrophobic interactions are minimized, nominally only hydrophilic and electrostatic interactions can be linked to the salt-induced solubility profiles; here β-C is totally soluble at all calcium concentrations and α_{s1}-B and α_{s1}-A were now found to have salting-in parameters, k_2 and m, of 2.5 liters · mol^{-1} and 4, and 11 liters · mol^{-1} and 8, respectively. α_{s1}-A is more readily salted-in and studies on the variation of S_1 with added KCl for this protein at 1°C indicated that salting-in is also mainly electrostatic in nature and may result from competition between K^+ and Ca^{2+} for binding sites rather than from solute–solvent interactions as previously proposed. Comparison of k_1 and k_2 values between the two genetic variants, coupled with the known sequence differences (the A variant is a linear deletion of 13 amino acids) suggest the existence of a hydrophobically stabilized ion pair in α_{s1}-B which is deleted in α_{s1}-A; it is speculated that such bonds may play a role in other calcium-induced changes in protein solubility.

Selective binding of calcium to proteins such as calmodulin regulates a variety of intracellular processes (1). These calcium–protein interactions characteristically occur with relatively high association constants. In contrast, many secretory and extracellular processes occur in the presence of relatively high concentrations of calcium, and under these conditions binding of calcium to proteins can occur with weaker affinity sites. Examples of these latter cases include dentin and bone formation (2), as well as saliva (3) and milk secretion (4). Although the ca-

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seins of milk occur as large colloidal aggregates which serve to transport inorganic calcium and phosphate, the individual purified components exhibit differing solubilities with regard to Ca^{2+} (for a review see Ref. (5)).

Proteins in solution are polyions and can be solubilized by low salt concentrations; however, as noted by Tanford (6) at higher salt concentrations (~1 M) salting-out can occur. Calcium is effective in salting-out processes; however, in the case of some proteins, particularly the phosphorylated proteins α_{s1}- and β-casein and other phosphoproteins, lower concentrations of Ca^{2+} (10 to 20 mM) cause changes in solubility, perhaps indicating a more selective role for this type of calcium binding (2, 5). In order to better understand these calcium-protein interactions, calcium-induced solubility profiles of selected purified caseins were reinvestigated. The data were analyzed with respect to models which are derived from the principles of thermodynamic linkage. What is linked in this case is the solubility and known calcium binding of the caseins.

EXPERIMENTAL PROCEDURES

Materials. Purified caseins were prepared as previously described (7) by DEAE-cellulose chromatography. Stock $CaCl_2$ solutions were prepared and their concentrations checked by atomic absorption analysis. The nomenclature of the genetic variants and abbreviations for casein are as previously described (8).

Solubility of caseins. The solubility of caseins at each of two temperatures, 1 and 37°C, was measured as follows:

(i) Dissolve caseins (about 20 mg/ml) in water and adjust pH to 7.0 with 0.1 *N* KOH or NaOH. Equilibrate in water bath at desired temperature for 15 to 20 min.

(ii) To 2 ml of protein solution (in thick-walled centrifuge tubes), blow in 2 ml of $CaCl_2$ solutions, with or without buffer ± KCl, invert the tube, let stand at desired temperature for 30 min.

(iii) Centrifuge for 15 min at 43,800g_{max} at desired temperature in a Beckman Model L-8 ultracentrifuge with an SW 60 Ti swinging bucket rotor.

(iv) Transfer 500μl of supernatant to a 5 ml volumetric flask containing 1 ml 1 N sodium citrate plus a few milliliters of water; make up to volume with water. When solubility is determined at 1°C, pipettes must be prechilled to avoid precipitation of protein in the pipette. Read in 1-cm cuvettes at 280 nm. Extinction coefficients, ϵ, 1 cm 1% 280 nm for α_{s1}-A[2] and -B are 10.0 and 4.7 for β-casein C (8).

Theory and data analysis. Characteristic changes in solubility of individual caseins occur as a function of calcium ion content (5). In addition these individual caseins bind calcium with well-documented association constants. Here we have thermodynamically linked the free energy of salt binding to the solubility of the various liganded species using the concept of Wyman's linked functions (9). For the biphasic behavior observed, we assumed two classes of binding sites. We next assumed that the following equilibra occur,

$$\underset{(S_0)}{p} + nx \overset{k_1^n}{\leftrightarrows} \underset{(S_1)}{PX_n} + mx \overset{k_2^m}{\rightleftarrows} \underset{(S_2)}{PX_nX_m}, \qquad [1]$$

where p is the unbound protein, x is the free salt, n and m are the number of X moles bound to species PX_n and PX_nX_m, and S_o, S_1, and S_2 are the solubilities of the liganded species indicated. For this study S_1 and S_2 will be relative to S_0. The mathematical relationship representing the above stoichiometry can be represented according to

$$S_{app} = S_0 f(p) + S_1 f(PX_n) + S_2 f(PX_nX_m), \qquad [2]$$

where S_{app} is the apparent protein solubility at a given salt concentration (X_T), $f(i)$ are the protein fractional components of species i, and the S's are species previously defined. Incorporation of the salt binding equilibrium constants (k_1 and k_2) as defined by [1] into [2] yields

$$S_{app} = \frac{S_o p}{p + k_1^n p x^n} + \frac{S_1 k_1^n p x^n}{p + k_1^n p x^n} + \frac{(S_2 - S_1) k_2^m p x^m}{p + k_2^m p x^m}, \qquad [3]$$

where p is the concentration in percentage of the unbound proteins and x is the concentration of unbound salt. Cancellation of common terms yields

$$S_{app} = \frac{S_0}{1 + k_1^n x^n} + \frac{S_1 k_1^n x^n}{1 + k_1^n x^n} + \frac{(S_2 - S_1) k_2^m x^m}{1 + k_2^m x^m}. \qquad [4]$$

It should be stressed here that the above expression is valid for sequential binding, i.e., $k_1 > k_2$, and n sites saturate prior to the binding of m sites on the protein, and for simplicity that n and m do not interact. Also, for n or m greater than one, k_1 and k_2 represent an average value for each class of the n or m binding sites. In reality n or m moles of salt will bind with only on equilibrium constant (K_1), i.e., $K_1 = k_1^n$ and $K_2 = k_2^m$.

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[2] Abbreviations used: α_{s1}-A, α_{s1}-casein A; α_{s1}-B, α_{s1}-casein B; β-C, β-casein C; RMS, root mean square; ATGEE, acetyltetraglycine ethyl ester.

Now, since the total salt concentration, X_T, is the sum of the free salt concentration, x, and the concentration of the bound salt of both species PX_n and PX_nX_m, it can be shown that

$$X_T = x\left(1 + \frac{nk_1^n P_T x^{(n-1)}}{1 + k_1^n x^n} + \frac{mk_2^m P_T x^{(m-1)}}{1 + k_2^m x^m}\right), \quad [5]$$

where P_T is the total concentration of protein. From Eq. [5] it can be seen easily that $X_T = x$ when P_T is small relative to x. For these experiments this assumption is reasonable because of the concentration range of the total salt and the molecular weight of the caseinates. Therefore, since the total salt concentration could be used in [4] instead of the free concentration; salt-induced solubility profiles were directly analyzed using a Gauss-Newton nonlinear regression analysis program developed at this laboratory by Dr. William Damert. All solubility profiles were analyzed by fixing the values of n and m and calculating the best least-squares fit for the optimum elevated k_1 and k_2 values. The n and m values were then fixed to new values and the whole procedure was repeated. The n and m values which yielded the minimum root mean square (RMS) and lowest error values for k_1 and k_2 were then reported.

RESULTS

Solubility at 37°C. Solubility determinations of α_{s1}-caseins A and B (α_{s1}-A, α_{s1}-B) and β-casein C (β-C) were performed at 37°C in 10 mM imidazole-HCl, pH 7.0, 0.07 M KCl, at initial protein concentrations of 10 mg/ml. As in the experiments of Noble and Waugh (10) the proteins precipitate when added $CaCl_2$ exceeds 5 mM (Figs. 1a and 1b). Creamer and Waugh (11) had suggested that about 13 sites of similar calcium binding strength exist in the α_{s1}-B at pH 6.6 and that when calcium ion concentration exceeds this critical binding level, precipitation occurs. Comparison of the solubility profiles of α_{s1}-A and -B indicates that at 37°C α_{s1}-A is more soluble than α_{s1}-B, while β-C is the most soluble. In order to quantify the data, nonlinear regression analyses were performed. The data of Fig. 1a were fitted by the first two terms of Eq. [4] since only monophasic behavior was observed. Values of k_1, were obtained at fixed integer values of n; the correct value of n was taken to be the fit with the minimum RMS. Figure 1a shows the fit to $n = 2$, 4, and 8 for α_{s1}-A; values for $n = 8$ gave the minimum RMS with the lowest error in k_1. Analysis of the solubility profiles of α_{s1}-A, α_{s1}-B, and β-C at 37°C, where hydrophobic interactions are maximized, showed no salting-in behavior so that k_2 and m were essentially zero. Values obtained for k_1 (salting-out) and n are given in Table I.

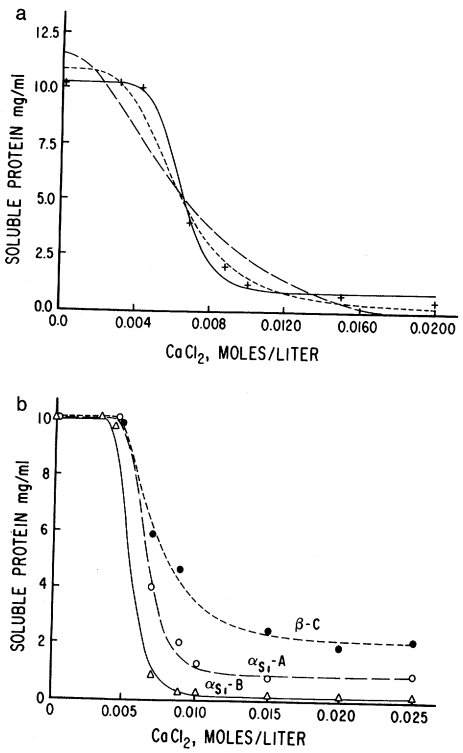

FIG. 1. Solubility at 37°C of the calcium salts of α_{s1}-caseins A and B, and β-casein C as a function of increasing $CaCl_2$ concentration. Solutions buffered at pH 7.0, 10 mM imidazole-HCl with 0.07 M KCl. (a) The experimental data for α_{s1}-A were fitted by Eq. [4] by nonlinear regression analysis with values of 2 (- -), 4 (---), and 8 (—) assigned to n. The best fit was obtained for $n = 8$. (b) Similar fits for α_{s1}-B and and β-casein C.

Solubility at 1°C. Figure 2 shows that β-casein C is not precipitatd at 1°C by Ca^{2+} at concentrations of up to 400 mM. It is known that hydrophobic forces are dominant in the association reactions of β-caseins (12). The solubility of β-casein C clearly distinguishes it from α_{s1}-B; it is known that β-casein binds Ca^{2+} at 1°C, but in this case binding is not linked to changes in solubility. Aliquot addition of calcium chloride solutions to α_{s1}-casein

TABLE I

CALCIUM-INDUCED INSOLUBILITY OF CASEIN AT 37°C[a]

Casein	k_1 (liters/mol)	n	S_1[b] (mg/ml)
α_{s1}-A	157 ± 3	8	0.9 ± 0.2
α_{s1}-B	186 ± 3	8	0.1 ± 0.1
β-C	156 ± 12	4	2.0 ± 0.3

[a] Solutions buffered at pH 7.0, 10 mM imidazole-HCl, 0.07 M KCl.

[b] S_1 denotes the maximum value for soluble protein at elevated Ca^{2+} concentrations.

results in a rapid decrease in solubility from 8 to 50 mM, where the protein is almost totally precipitated. When the calcium chloride concentration exceeds 100 mM a gradual salting-in of the protein ensues at 1°C. The data for α_{s1}-B were fitted by Eq. [4] and the salting-out parameters k_1 and n, and the salting-in parameters k_2 and m were determined (Table II). The α_{s1}-A genetic variant, in contrast to α_{s1}-B, exhibits extraordinary solubility behavior over a broad range of calcium chloride concentrations. At 1°C (Fig. 3) α_{s1}-A, like α_{s1}-B (Fig. 2), is precipitated with calcium at about 8 mM, whereupon the net electrical charge on the protein may be close to zero. In the absence of electrolyte (KCl) or buffer, and after aliquot addition of $CaCl_2$, the protein is driven into solu-

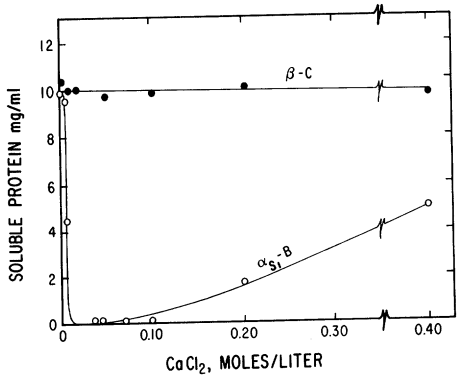

FIG. 2. Solubility at 1°C of calcium α_{s1}-B caseinate and calcium β-C caseinate as a function of increasing $CaCl_2$ concentration at 1°C. Data were fitted by Eq. [4].

TABLE II

CALCIUM-INDUCED INSOLUBILITY AND SOLUBILITY OF CASEINS AT 1°C[a]

Casein	k_1 (liters/mol)	n	k_2 (liters/mol)	m
α_{s1}-B	123 ± 5	8	2.5 ± 0.2	4
α_{s1}-A	68 ± 1	8	10.6 ± 0.3	8
β-C	Totally soluble			

[a] Conditions as in Table I.

tion at 90 mM. The protein is now positively charged, acting as a cation. This conclusion was verified by free-boundary electrophoresis at pH 7.0, 10 mM imidazole, 150 mM $CaCl_2$, where the protein is soluble at 1°C; it migrates (+1.36 $cm^2\ V^{-1}\ s^{-1} \times 10^{-5}$) toward the cathode (7).

Influence of electrolyte on salting-out and salting-in constants. Aliquot addition of $CaCl_2$ in the presence of varying concentrations of KCl results in a shift in the solubility profile of α_{s1}-A at 1°C (Fig. 3). KCl was chosen as the electrolyte because it occurs at a higher concentration than NaCl in milk serum (5). The data were analyzed and fitted by Eq. [4]; parameters k_1, k_2, n, and m are given in Table III. Note that as the KCl concentration increases, n drops to 4 for Ca^{2+}; the protein apparently requires less bound Ca^{2+} to precipitate. On the other hand, m for resolubilization remains at 8. Increasing ionic strength by the addition of KCl for each calcium-induced solubility profile of α_{s1}-A at 1°C decreased k_1 and k_2 values. The variations of k_1 and k_2 with KCl are given in Figs. 4a and 4b. For k_1, a nearly monotonic decrease occurs; Fig. 4a can be analyzed as a potassium-binding isotherm itself and an association constant K_{a1} computed for KCl-protein interactions. The value given in Table IV, line 1, was found to be 20 ± 6 liters · mol^{-1}. For k_2, the variation appeared more complex with two transitions. Analyzing these data K_{a2} and K'_{a2} were calculated (Table IV, lines 2 and 3). As compared with the primary k_1 values for Ca^{2+} precipitation, all these derived constants related to KCl effects are substantially smaller. Changes are consistent with the electrostatic character of these

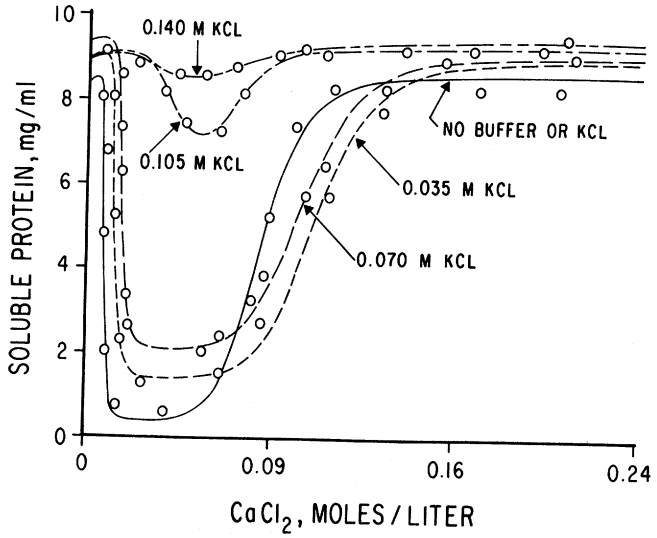

FIG. 3. Solubility at 1°C of calcium α_{s1}-A caseinates as a function of increasing $CaCl_2$ and KCl concentrations. Data were fitted by Eq. [4]. Results are in Table III.

binding isotherms and could be attributed to a competitive effect of elevated potassium ion for calcium binding sites or to KCl-solvent interactions affecting solubility. The overall difference for the behavior of α_{s1}-A relative to α_{s1}-B, however, must reside in the structural differences due to the deletion mutation in the A variant.

Influence of electrolyte on soluble protein (S_1). Further insights into the source of the effects resulting in the α_{s1}-A solubility profiles can be obtained by analysis of the

TABLE III

IONIC STRENGTH DEPENDENCE OF CALCIUM-INDUCED SOLUBILITY OF α_{s1}-CASEIN A AT 1°C

KCl (mM)	k_1[a]	k_2[a]	S_1[b] (mg/ml)
—	130 ± 3	13.3 ± 0.3	0.4 ± 0.2
35[c]	82 ± 1	10.1 ± 0.1	1.4 ± 0.1
70[c]	68 ± 1	10.6 ± 0.2	2.1 ± 0.1
105[d]	22 ± 1	15.9 ± 0.1	4.7 ± 0.4
140[d]	34 ± 1	15.7 ± 0.2	8.4 ± 0.1

[a] Liters/mol.

[b] S_1 denotes the maximum value for soluble protein after precipitation but before total resolubilization.

[c] $n = 8$ and $m = 8$.

[d] $n = 4$ and $m = 8$.

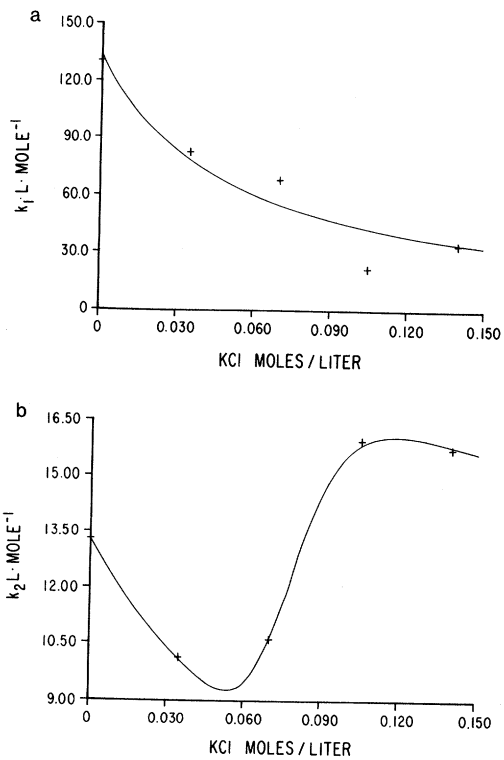

FIG. 4. Variations of k_1 (a) and k_2 (b) with KCl concentration. These parameters obtained from analysis of Fig. 3 (Table III) were fitted by Eq. [4]; results are in Table III.

TABLE IV

COMPARISON OF DERIVED CONSTANTS FROM THE EFFECT OF KCl ON k_1, k_2, AND S_1 FROM THE CALCIUM-INDUCED SOLUBILITY OF α_{s1}-A AT 1°C

Derived constant	Source of constant	Value (liter/mol)	n	log K
K_{a1}	k_1[a]	20.0 ± 6.0	1	1.30
K_{a2}	k_2-1st Transition[b]	9.2 ± 0.6	1	0.96
$K'_{a2'}$	k_2-2nd Transition[b]	12.5 ± 0.5	8	1.10
K_{as}	S_1-1st Transition[c]	15.9 ± 0.8	1	1.20
$K'_{as'}$	S_1-2nd Transition[c]	8.9 ± 0.3	8	0.94
K_a^-	$K^+ + HPO_4^{-2}$[d]	19.9	1	1.30

[a] See Fig. 4a.
[b] See Fig. 4b.
[c] See Fig. 6.
[d] Sillen and Martell (22).

variation of the parameter S_1 of Table III. This term is taken to represent the soluble form of α_{s1}-A found in the valley between 2 and 8 mM $CaCl_2$ (Fig. 3). It was attempted to relate the degree of solubilization by KCl with the surface free energy of the protein. Change in surface at the water-protein interface with surface tension of solvent, as given by Arakawa and Timasheff (13), is

$$(\partial\mu_{Pr}/\partial m_x)_{T,P,m_{Pr}} = NS_{Pr}(\partial\sigma/\partial m_x)_{T,P,m_{Pr}} \quad [6]$$

where Pr = protein, x = ligand, σ = surface tension of salt solution, N = Avagadro's number, and S_{Pr} = surface area of the protein. The calculated variation of surface tension with salt has been reported (14) and the slope $\partial\sigma/\partial m_x$ is equal to 0.06889 at 0.15 M KCl. If we take the ratio of S_1 at varying concentrations of KCl to S_1 at zero KCl to be equivalent to the change in chemical potential of the protein with salt then a plot of the natural log of this ratio versus salt (Fig. 5) gives a slope of 1.308. Substitution of these two slopes into Eq. [6] gives an estimate of the surface area (S_{Pr}) of the soluble protein. This value is 7193 ± 1076 Å^2; for an equivalent sphere this would yield an R_G of 18 Å and a molecular weight of 21,600 using the relationship of 9.42 M$^{2/3}$ (15). The sequence molecular weight of α_{s1}-A is 22,068. If this analysis holds, the implication is that at 1°C, in the absence of hydrophobic associations, salting-in of the whole protein, not just a hydrophobic portion, is accomplished either by KCl-solvent effects (for which the sign of the slope is incorrect) or by salt (either K^+ or Cl^-)-protein interactions.

Another approach to analyzing this salting-in effect (increased S_1 with increased KCl) is to use the Setschenow equation as defined by Robinson and Jencks (16):

$$\log Pr_0/Pr = K_sC_s. \quad [7]$$

Here Pr_0 and Pr are the molar solubilities of protein in water and salt, respectively, K_s is the Setschenow constant (which is negative for salting-in and positive for salting-out), and C_s the molar concentration of salt. This equation is identical to Fig. 5 except for the inverse ratio of protein and nature of the log. K_s for α_{s1}-A is compared to the values found by Robinson and Jencks for acetyltetraglycine ethyl ester (ATGEE) a neutral protein analog with no formal change (Table V). It can be seen that the slope, K_s, is indeed negative, which is opposite in sign to the effect of KCl on ATGEE. In addition its value for α_{s1}-A is larger by far than that observed for the salting-in through weaker charge-dipole interactions which occur between ATGEE and LiI or $CaCl_2$ (16).

Since the magnitude of salting-in for S_1 is larger than expected for weaker salt-solvent forces, it was decided to analyze the variations of S_1 with KCl as a binding

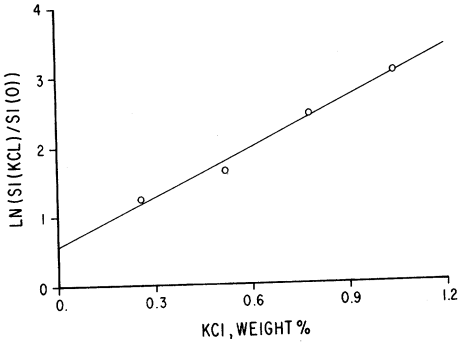

FIG. 5. Effect of KCl concentration on change of solubility of protein (α_{s1}-A) at 1°C relative to solubility in the absence of KCl.

TABLE V

COMPARISON OF SETSCHENOW SALTING-OUT CONSTANTS (K_s) FOR α_{s1}-A AND ACETYLGLYCINE ETHYL ESTER (ATGEE)

	K_s	Salt	Ref
α_{s1}-A	−7.411	KCl	a
ATGEE	−0.280	LiI	b
	−0.090	$CaCl_2$	b
	+0.046	KCl	b
	+0.450	$(NH_4)_2SO_4$	b

Note. Ref. (a) this study; and (b) Robinson and Jencks (16).

isotherm (Fig. 6). Two cooperative transitions appear to occur. These were analyzed in terms of Eq. [4] and values of K_{as}, K'_{as}, *and* n were calculated; these derived constants are compared with those derived from the variance of k_1 and k_2 with KCl in Table IV. All of the constants derived from k_2 or S_1 appear to be self-consistent in order of magnitude and in effect (salting-in), and are in the order of magnitude of K_a for $K^+ + HPO_4^{-2}$. The most readily apparent conclusion is that salting-in in this particular case may be the result of direct salt (perhaps both anion and cation) interactions with charged groups (possibly phosphates) of the protein. This effect may have somewhat wide ranging implication for calcium-protein-salt interactions.

Effect of various cations at 1°C. Figure 7 illustrates the solubility of α_{s1}-A in the presence of various cations. Cu^{2+} and Zn^{2+} are the most effective precipitants, as might be expected from their atomic numbers. Coordinate complexes may be formed between α_{s1}-A molecules with Cu^{2+} and Zn^{2+} and Co^{2+}. Ca^{2+} is effective, as a precipitant, to a lesser extent than Cu^{2+} or Zn^{2+}, whereas Mg^{2+} is the least effective of the five cations studied. The salting-out and salting-in constants were estimated for each cation and are given in Table VI. Cation variation (i.e., use of magnesium, calcium, cobalt, copper, and zinc) of these profiles showed k_1 and k_2 behavior consistent with concepts of phosphate- and carboxylate-ligand coordination, respectively. Clearly, an inverse relationship exists between casein solubility (as quantified by changes in k_1) and the atomic number of the divalent cations studied. The salting-in constant k_2 appears to decrease and then increase with atomic number; no apparent correlation with ionic radius is evident.

DISCUSSION

Choice of model. The caseins of milk can be subdivided on the basis of their solubility in Ca^{2+} solutions with α_s- and β-caseins being calcium insoluble and κ-casein being calcium soluble (5). The experiments performed in this work measure the concentration of soluble protein as a function of added calcium ion. In earlier works Waugh and co-workers (17, 18) established that under these conditions the various species of protein present are in thermodynamic equilibrium. In addition, under identical environmental conditions, calcium ion binding to the caseins has been demonstrated by equilibrium dialysis (19). Equation [1], cast in the form of a binding isotherm equates the proposed stoichiometric terms (p, PX_n, PX_nX_m) with the soluble species designated S_0, S_1, and S_2, respectively. That ligand binding can change solubility, has much precedence in the literature (13, 16). Early studies on solubility (6) linked the chemical potential of protein solutions with electrostatic effects; these latter studies were primarily concerned

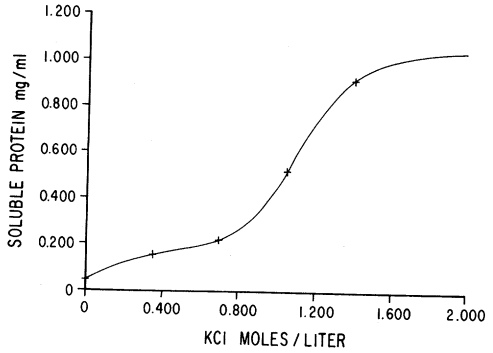

FIG. 6. Effect of KCl on S_1, the amount of soluble protein after initial calcium-induced precipitation. Data were analyzed by Eq. [4]; results are in Table IV.

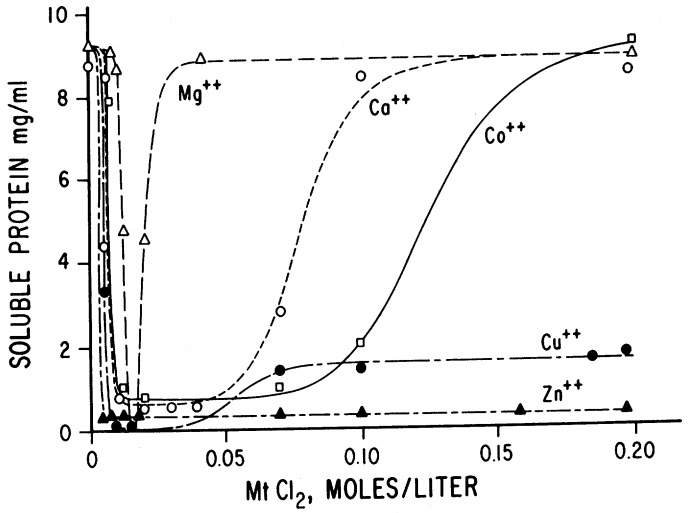

FIG. 7. Solubility at 1°C of various salts of α_{s1}-A caseinates as a function of increasing concentration. Data were fitted by Eq. [4].

with proton (H^+) binding. Here a similar linkage may occur with Ca^{2+} binding.

From a mathematical point of view the solubility profiles studied are either monophasic (Fig. 1) or biphasic (Figs. 3 and 7). Many proposed equations for solubility (including those of Melander and Horvath (14)) were tested but could not be fit with the data. The general equation derived in this study (Eq. [4]) can be used to quantitate either monophasic behavior (Fig. 1) by use of terms 1 and 2, or biphasic behavior using terms 1 through 3. In the latter case two consecutive events must be postulated since solubility first decreases and then increases. Fits to equations with extended terms were unsatisfactory.

Analyses of the solubility profiles of two genetic variants of the major bovine casein (α_{s1}-) and β-casein have been accomplished using Wyman's theory of thermodynamic linkage. These analyses have yielded parameters which quantify the changes in solubility. The parameters k_1 and k_2 are postulated to be related to average association constants for the binding of calcium to casein. The logic behind this assumption is that as the casein

TABLE VI

CATION-INDUCED SOLUBILITY OF α_{s1}-CASEIN A AT 1°C[a]

Cation	k_1[b]	k_2[b]	$(S_2\text{-}S_1)$[c] (mg/ml)	Atomic No.	R[d] (Å)
Mg^{2+}	76 ± 9	56 ± 7	8.4 ± 1.3	12	0.66
Ca^{2+}	150 ± 27	13 ± 2	8.2 ± 1.4	20	0.99
Co^{2+}	166 ± 4	8.1 ± 0.2	8.5 ± 0.2	27	0.72
Cu^{2+}	229 ± 2	18 ± 2	1.5 ± 0.1	29	0.72
Zn^{2+}	373 ± 27	202 ± 35	0.20 ± 0.03	30	0.74

[a] $n = m = 8$ for all calculations.

[b] Liters/mol.

[c] $(S_2\text{-}S_1)$ = concentration of soluble α_{s1}-A in milligrams per milliliter.

[d] Cation atomic radius in angstrom.

binds Ca^{2+} a charge neutralization occurs, the complex approaches its isoelectric point, and precipitation occurs; increased binding may cause charge reversal leading to increased solubility. Previous research by Waugh and co-workers (17, 18) has indicated that all of the species present during aliquot precipitation studies are in true equilibrium, so that analysis of the soluble protein as a function of total calcium ion present should yield parameters (k) akin to association constants for calcium binding, provided that the binding is linked to a change in the concentrations of soluble species. The events are analogous to proton binding causing increased solubility on either side of the isoelectric point of a protein (6); here Ca^{2+} replaces H^+. Table VII shows a comparison of experimental values for Ca^{2+} binding compiled by Dickson and Perkins (19) with values (log k_1, k_2) obtained in this study. The k_1 values are in good agreement with literature values for calcium binding from dialysis experiments for the caseinates and for binding to model phosphate compounds. On the other hand, salting-in constants (k_2) appear to be more in agreement with association constants related to Ca^{2+} binding to isolated carboxylate groups. Thus, salting-in may be the result of further Ca^{2+} binding to carboxyl groups resulting in increased positive charge and solubility.

TABLE VII

COMPARISON OF ASSOCIATION CONSTANTS FOR VARIOUS CALCIUM COMPLEXES WITH VALUES OF k_1 AND k_2

Complex of Ca^{2+}	log k_a[a]	log k_1[b,c]	log k_2[b,c]
α_{s1}-casein B	2.6	2.26 ± 0.04	0.39 ± 0.18
α_{s1}-casein A	—	2.20 ± 0.04	1.02 ± 0.06
β-casein	2.6	2.26 ± 0.18	—
O-Phosphoserine	2.2		
Glutamate	0.8		
Acetate	0.6		

[a] From the data of Dickson and Perkins (19).

[b] This study; k_1 from Table I, k_2 from Table II.

[c] Errors for log k's estimated from $\Delta \log = 2.303 \times \Delta k/k$.

The second parameter obtained by this method of analysis is n or m. In the case of n the values obtained (8 and 4 for α_{s1}- and β-caseins) correlate with the number of moles of calcium bound as calculated from the data of Dickson and Perkins ((19) Table VII) at these conditions of pH and ionic strength. Other values can be obtained outside of the normal physiological ranges (20). Coincidently, the values of n also correspond to the number of phosphate residues found by sequence for the respective caseinates. (Note β-C has only 4 groups as opposed to 5 found on A and B forms; Ref. (8)). The net charge on the α_{s1}-B molecule at neutral pH can be calculated to be −26; binding of eight divalent calcium ions would decrease this number to −10, thus considerably reducing the molecule's net charge but not bringing about total charge neutralization. In a similar fashion the net charge on β-casein C is −12 and binding of four divalent cations reduces this number to −4. The term n reflects only that binding linked to changes in solubility. Other binding not linked to solubility changes could occur but be undetected; e.g., binding to higher affinity sites could occur first followed by the solubility linked binding. Creamer and Waugh (11) calculated that ~13 mol of Ca^{2+} were bound to α_{s1}-B at precipitation. This could be accounted for by 5 carboxylates and 8 phosphates but analysis here cannot discriminate between the choice of carboxylates with higher than normal affinity for Ca^{2+} and phosphates. It is interesting to note that in the case of α_{s1}-casein A, which is totally resolubilized at 1°C, $n + m = 16$, reflecting a charge reversal to a cationic species with a net charge of +8. In fact, free boundary electrophoresis in calcium showed the α_{s1}-A molecule to have a cathodic mobility, and equilibrium dialysis experiments gave a value of 17 mol of Ca^{2+} bound at 1°C in 0.07 M KCl (7). It thus appears that the parameters n and m are linked to the number of mol of Ca^{2+} bound per monomer which lead to changes in solubility, while the values of k_1 and k_2 may relate to the association constants for phosphate and/or carboxylate binding of Ca^{2+} respectively. It must be remembered

that k_1 and k_2 are average constants reflecting binding under specific conditions which results in solubility changes. This would probably be true for constants obtained by equilibrium dialysis (19). The latter workers showed that $\bar{\nu}$ ranged from 5 to 16 depending upon the ionic strength of the solution; values of $\bar{\nu}$ obtained at conditions comparable to the ones employed here are 8 and 5 for α_{s1}- and β-caseins (Table I). Because of the role of ionic strength in these multiple equilibria, it was felt that the study of solubility changes at 1°C for α_{s1}-A might clarify this situation.

In studying salting-out of proteins, Melander and Horvath (14) attempted to account for effects of salts through a combination of electrostatic and hydrophobic interactions. The electrostatic changes in solubility were interpreted on the basis of classical theory and correlated with a salting-in term Λ. On the other hand hydrophobic free energy changes were correlated, through effects of salt on surface tension of the medium, with salting-out. The summation of these two opposite terms thus accounts for the salt solubility of a protein. Their work was a further extension of the solvophobic theory of Sinanoglu (21). This latter theory has become known as cavity theory because it breaks down ΔG of solution of a molecule into the energy necessary to create a cavity in the solvent as well as the sum of the interactions between solute and solvent which include electrostatic, van der Waals, and (as modified by Melander and Horvath) hydrophobic interactions. These latter workers emphasized the importance of the molal surface tension increment in depressing cavity formation in hydrophobically driven salting-out. The magnitude of the molal surface tension increment (σ) was correlated with the salting-out properties of ions. Since both KCl and $CaCl_2$ have positive σ values, according to this theory one would expect the salting-out seen in Fig. 1 to be the result of hydrophobic interactions driven by salt-induced changes in surface tension. At 1°C, β-casein, whose associative properties are nearly purely hydrophobic in nature, is completely soluble (Fig. 2); this seems to fit the solvophobic theory well. However, α_{s1}-B is still precipitated and is salted-in at higher $CaCl_2$ concentrations (Fig. 2). Since hydrophobic interactions are repressed at 1°C, salt binding and/or charge interactions must account for the precipitation phase of the calcium-induced solubility profile under these conditions. Increased solubility at elevated $CaCl_2$ is qualitatively as expected from the salting-in portion of the Melander and Horvath theory.

Analysis of the curves of Fig. 3, allows for the calculation of an additional parameter S_1. This parameter represents the soluble form of the caseinate found in the valley between 2 and 8 mM $CaCl_2$. The increase in S_1 with increased KCl (Table III) was first correlated with the incremental change in surface tension through Eq. [6]. It was calculated that the surface area of the soluble protein, as well as R_G and molecular weight, were in agreement with the values expected for monomeric casein. It must be born in mind that Eq. [6] is a partial differential equation and as such it is difficult to integrate unless the proper conditions are met experimentally and theoretically. Care should be taken so that the proper state of the protein (S_1 in this case) be maintained when increasing the surface tension with salt to test cavity theory. Since the surface evaluated for α_{s1}-A at 1°C, where hydrophobic interactions are minimized, is in agreement with that of a monomeric protein (rather than the hydrophobic surface alone) and since the effect is opposite in sign from that predicted by Melander and Horvath (14) [KCl should always decrease S_1 (Table III)]. We conclude that solvent dispersive effects due to increased internal pressure are not the primary forces at work here. We must therefore look elsewhere for the source of the effects seen here in terms of S_1.

Robinson and Jencks (16) studied the effects of salts on the solubility of ATGEE. They concluded that the effects of salt solutions on proteins may be accounted for on the basis of the effects of ions on peptide and amide groups which

become exposed to solvent when a protein undergoes a change in physical state. It seemed as though their ideas would be applicable here in the question of the increased solubility of α_{s1}-A by KCl, that is, a change from insoluble to a soluble state. Robinson and Jencks (Table V) demonstrated that the activity coefficient of ATGEE, as approximated by Pr_0/Pr (Eq. [7]), was increased by KCl resulting in salting-out, while $CaCl_2$ resulted in salting-in. They found an excellent correlation between the effects of a salt on the solubility of ATGEE and its ability to salt-out proteins. Robinson and Jencks favored direct interaction of amide groups with salt as the major, though not sole, force in salting-out of ATGEE. For KCl-α_{s1}-A the trend is reversed (salting-in occurs). In addition the size of K_s for salting-in of ATGEE by $CaCl_2$ and Li is much lower than that observed for salting-in of α_{s1}-A by KCl, indicating that stronger forces are involved here (Table V).

Arakawa and Timasheff (13) studied the preferential interaction of solvents with proteins in concentrated salts; they concluded that a distinct relationship exists between surface tension, solubility, and preferential interactions. They suggested that increased surface tension should result in exclusion of contact between salt and protein. This is the reverse of what would be expected from experimental observation since their work on preferential hydration of proteins by salts correlated more with the results of Robinson and Jencks (16) on ATGEE solubility. For salting-in, then, they reasoned that some attractive forces between salt and protein exist and that these attractive forces would overcome the repulsive forces due to increased surface tension. From the considerations presented above it seems as though salting-in behavior observed here should be correlated more with direct interactions rather than dispersive forces due to surface tension. In evaluating the changes occurring in S_1 with KCl, for α_{s1}-A it could thus be argued that stronger attractive forces are at work in salting-in. When the variation of S_1 with KCl was treated as a simple binding isotherm, a two phase system emerged: one class of sites more readily binds K^+ perhaps competing with Ca^{2+}, and then a second class ($n = 8$) results in nearly complete solubilization (Table IV and Fig. 6).

Further evidence for the relatedness of these parameters to salt binding comes from the variance of k_1 and k_2 with added KCl for α_{s1}-A at 1°C. As seen in Fig. 4a, increased KCl decreases k_1 as the K^+ competes with Ca^{2+} and the "apparent" association constant for Ca^{2+}-protein decreases. Treating the variation of k_1 with KCl as a binding isotherm for K^+ yields a log K_{a1} of 1.3 which is quite close to the association constant for HPO_4^{-2} and K^+ (log $K = 0.8$; Sillen and Martell (22)). For k_2 the variation is somewhat more complex; at first some competition between Ca^{2+} and K^+ may decrease salting-in, but then (perhaps after saturation of one class of sites) the salting-in is facilitated (Fig. 4b). Estimations of k_1 and k_2 for effects of salts other than Ca^{2+} argue for the same relative binding with k_1 being $> k_2$ and in the same order expected for phosphate and carboxylate binding, respectively (Table VI). When the values of the derived association constants for these interactions are compared with those derived from the variance of S_1 with KCl, it appears as though the salt (and this could mean both K^+ and Cl^-) is interacting directly with the charged groups on the protein to increase solubility. The implication is that the solubility of the protein is increased by KCl (or NaCl) at 1°C even in the presence of relatively high calcium concentrations. While this was not the general case for proteins at 37°C it points to the potential solubilization by KCl and/or NaCl of calcium-precipitated proteins if the total calcium levels in a system are reduced (for casein <5 mM). Such forces do disrupt casein micelles (12) and could play a role in bone resorption and perhaps tooth regression. Thus complex multiple equilibria occur in these fluids and the total ionic environment participates in these processes and cannot be neglected. The derived K_a's when viewed as dissociation constants (60 to 100 mM) are in the physiological range for the total $Na^+ + K^+$ in

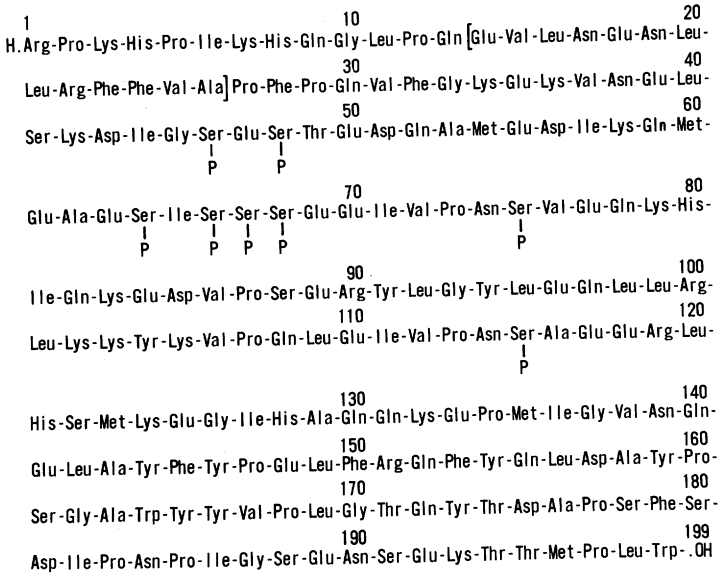

FIG. 8. Sequence of α_{s1}-casein B showing amino acids deleted to yield the α_{s1}-A variant (8).

extracellular fluids; for example, Na^+ and K^+ are 20 and 39 mM, respectively, in cow's milk (5). The values of all of these derived K_a's are also of the order of magnitude expected for K^+ binding to HPO_4^{-2} (Table IV). This interpretation is in agreement with the salting-in observations of Robinson and Jencks (16) on ATGEE and with the observations on preferential hydration of Arakawa and Timasheff (13).

The curves for the α_{s1}-A calcium complexes show a strong tendency toward resolubilization at 1°C, whereas α_{s1}-B does not. α_{s1}-A represents a sequential deletion of 13 amino acid residues (Fig. 8). Note that residues 14 and 18 are glutamic acids, while residue 22 is an arginine. The effects seen here are not the result of simple charge reduction. Since the remainder of the amino acid residues deleted are hydrophobic or noncharged, it is possible that a strong hydrophobically stabilized intramolecular ion pair occurs in α_{s1}-B resulting in a precipitate which is not readily salted-in. In the absence of this bond, α_{s1}-A can be salted-in. The genetic alteration produces a major change in the physical properties of the α_{s1}-A protein. Similar ion pairs may be of importance in other phosphoprotein systems such as dentin formation and plaque deposition. Examination of the sequences of salivary phosphopeptides shows repeating sequences of Arg and Glu interspersed with hydrophobic residues (23). Future research on site-specific changes in the caseins and their resulting changes in physical properties may be possible since Stewart and co-workers (24) have succeeded in constructing the cDNA for the α_{s1} gene.

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